

DE-CROSSLINKING COMPOUNDS AND METHODS OF USE FOR SPATIAL ANALYSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Application Serial No. 63/059,535, filed on
5 July 31, 2020, which is incorporated herein by reference in its entirety.

BACKGROUND

Cells within a tissue of a subject have differences in cell morphology and/or function
10 due to varied analyte levels (e.g., gene and/or protein expression) within the different cells. The specific position of a cell within a tissue (e.g., the cell's position relative to neighboring cells or the cell's position relative to the tissue microenvironment) can affect, e.g., the cell's morphology, differentiation, fate, viability, proliferation, behavior, and signaling and cross-talk with other cells in the tissue.

15 Spatial heterogeneity has been previously studied using techniques that only provide data for a small handful of analytes in the context of an intact tissue or a portion of a tissue, or provide a lot of analyte data for single cells, but fail to provide information regarding the position of the single cell in a parent biological sample (e.g., tissue sample).

Formaldehyde fixation is a common form of tissue preservation, and after
20 formaldehyde fixation, tissues are often embedded in paraffin wax (FFPE) for long term storage. Formaldehyde fixation crosslinks amine functional groups of nucleic acids and proteins through covalent linkage.

SUMMARY

25 Crosslinked amine functional groups (e.g., in aminated crosslinks) are typically not compatible with gene expression analysis, including spatial gene expression analysis. Traditionally, heat has been used to try to break the crosslinks of formaldehyde-fixed tissues. This disclosure is based on, at least in part, the identification and use of de-crosslinking reagents that are suitable for use with crosslinked biological samples for spatial analysis.

30 In one ~~aspect~~example, provided herein is a method of producing a de-crosslinked biological sample, the method including (a) contacting a fixed biological sample with a substrate including a plurality of capture probes, wherein a capture probe of the plurality of capture probes includes a capture domain, and (b) contacting the fixed biological sample with a de-crosslinking agent, thereby producing the de-crosslinked biological sample.

In one aspectexample, provided herein is a method of producing a de-crosslinked biological sample, the method including (a) contacting a fixed biological sample with a substrate including a plurality of capture probes, wherein a capture probe includes a capture domain, and (b) contacting the fixed biological sample with a de-crosslinking agent, thereby
5 producing the de-crosslinked biological sample.

In another aspectexample, provided herein is a method of producing a de-crosslinked biological sample, the method including (a) contacting a fixed biological sample with a de-crosslinking agent, thereby producing the de-crosslinked biological sample, and (b)
10 contacting the de-crosslinked biological sample with a substrate including a plurality of capture probes, wherein a capture probe of the plurality of capture probes includes a capture domain.

In another aspectexample, provided herein is a method of producing a de-crosslinked biological sample, the method including (a) contacting a fixed biological sample with a de-crosslinking agent, thereby producing the de-crosslinked biological sample, and (b)
15 contacting the de-crosslinked biological sample with a substrate including a plurality of capture probes, wherein a capture probe includes a capture domain.

In some embodimentexamples, the de-crosslinking agent can be a compound of Formula (I) or a compound of Formula (II). In some embodimentexamples, the de-crosslinking agent can be selected from the group consisting of compounds (1)-(18). In some
20 embodimentexamples, the de-crosslinking agent can be a compound of Formula (I). In some embodimentexamples, the de-crosslinking agent can be selected from the group consisting of compounds (1)-(14). In some embodimentexamples, the de-crosslinking agent can be selected from the group consisting of compounds (1)-(11). In some embodimentexamples, the de-crosslinking agent can be a compound of Formula (II). In some embodimentexamples, the
25 decrosslinking agent is selected from the group consisting of compounds (15)-(18). In some embodimentexamples, the de-crosslinking agent can be compound (1). In some embodimentexamples, the de-crosslinking agent can be contacted to the fixed biological sample at a concentration of about 10 mM to about 500 mM. In some embodimentexamples, the de-crosslinking agent can be contacted to the fixed biological sample at a concentration of
30 about 10 mM to about 100 mM. In some embodimentexamples, the de-crosslinking agent can be contacted to the fixed biological sample at a concentration of about 50 mM to about 150 mM. In some embodimentexamples, the de-crosslinking agent can be contacted to the fixed biological sample at a concentration of about 30 mM to about 70 mM. In some
35 embodimentexamples, the de-crosslinking agent can be contacted to the fixed biological sample at a concentration of about 40 mM to

about 60 mM. In some [embodimentexamples](#), the de-crosslinking agent can be contacted to the fixed biological sample at a concentration of about 50 mM.

In some [embodimentexamples](#), the step of contacting the fixed biological sample with the de-crosslinking agent can be performed for about 1 minute to about 120 minutes. In some [embodimentexamples](#), the step of contacting the fixed biological sample with the de-crosslinking agent can be performed for about 30 minutes to about 90 minutes. In some [embodimentexamples](#), the step of contacting the fixed biological sample with the de-crosslinking agent can be performed for about 60 minutes.

In some [embodimentexamples](#), the step of contacting the fixed biological sample with the de-crosslinking agent can be performed at a temperature of about 45 °C and about 95 °C. In some [embodimentexamples](#), the step of contacting the fixed biological sample with the de-crosslinking agent can be performed at a temperature of about 60 °C to about 80 °C. In some [embodimentexamples](#), the step of contacting the fixed biological sample with the de-crosslinking agent can be performed at a temperature of about 70 °C.

In some [embodimentexamples](#), the de-crosslinking agent can be present in a solution or a suspension. In some [embodimentexamples](#), the solution or suspension further includes a buffer. In some [embodimentexamples](#), the buffer can be selected from the group consisting of: tris(hydroxymethyl)aminomethane (Tris), tris(hydroxymethyl)aminomethane-Ethylenediaminetetraacetic acid (TE), phosphate-buffered saline (PBS), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 2-morpholin-4-ylethanesulfonic acid (MES), and a combination thereof. In some [embodimentexamples](#), the buffer can be present in the solution or the suspension at a concentration of about 5 mM to about 50 mM. In some [embodimentexamples](#), the buffer can be present in the solution or the suspension at a concentration of about 20 mM to about 40 mM. In some [embodimentexamples](#), the buffer can be present in the solution or the suspension at a concentration of about 30 mM.

In some [embodimentexamples](#), the fixed biological sample can be a paraffinized fixed biological sample. In some [embodimentexamples](#), method further includes, prior to the step of contacting the paraffinized fixed biological sample with the de-crosslinking agent, a step of deparaffinizing the paraffinized fixed biological sample, thereby producing a de-paraffinized fixed biological sample, and optionally, rehydrating the de-paraffinized fixed biological sample. In some [embodimentexamples](#), the step of deparaffinizing the paraffinized fixed biological sample includes contacting the paraffinized fixed biological sample with xylene and ethanol. In some [embodimentexamples](#), the step of rehydrating the de-paraffinized fixed biological sample includes contacting the de-paraffinized fixed biological sample with water. In some

embodimentexamples, the step of deparaffinizing the paraffinized fixed biological sample includes, contacting the paraffinized fixed biological sample with xylene, absolute ethanol, about 96% ethanol, and about 70% ethanol. In some embodimentexamples, the step of deparaffinizing the paraffinized fixed biological sample includes, sequentially, contacting the paraffinized fixed biological sample with xylene, absolute ethanol, about 96% ethanol, and about 70% ethanol.

In some embodimentexamples, the method further includes, prior to the step of contacting the fixed biological sample with the de-crosslinking agent, a step of pretreating the fixed biological sample. In some embodimentexamples, the step of pretreating the fixed biological sample includes contacting the fixed biological sample with a proteinase. In some embodimentexamples, the proteinase can be present in a solution or a suspension. In some embodimentexamples, the proteinase can be a collagenase. In some embodimentexamples, the proteinase can be present in the solution or the suspension at a concentration of about 0.05 to about 0.5 U/ μ L. In some embodimentexamples, the proteinase can be present in the solution or the suspension at a concentration of about 0.1 to about 0.3 U/ μ L. In some embodimentexamples, the proteinase can be present in the solution or the suspension at a concentration of about 0.2 U/ μ L. In some embodimentexamples, the solution or the suspension further includes a detergent. In some embodimentexamples, the detergent can be a non-ionic detergent. In some embodimentexamples, the detergent can be 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol. In some embodimentexamples, the detergent can be present in the solution or the suspension at a concentration of about 0.05% (v/v) to about 2% (v/v). In some embodimentexamples, the detergent can be present in the solution or the suspension at a concentration of about 0.1% (v/v) to about 1% (v/v). In some embodimentexamples, the detergent can be present in the solution or the suspension at a concentration of about 0.5% (v/v). In some embodimentexamples, the solution or suspension further includes a buffer. In some embodimentexamples, the buffer can be Hank's Balanced Salt Solution (HBSS) buffer. In some embodimentexamples, the buffer can be TE buffer. In some embodimentexamples, the buffer includes Tris, TE, PBS, HEPES, MES, or a combination thereof. In some embodimentexamples, the solution or the suspension has a pH of about 7.0 to about 9.0. In some embodimentexamples, the solution or the suspension has a pH of about 7.5 to about 8.5. In some embodimentexamples, the solution or the suspension has a pH of about 8.0.

In some embodimentexamples, the method further includes, after the step of contacting the fixed biological sample with the de-crosslinking agent, a step of permeabilizing the de-crosslinked biological sample.

In some embodimentexamples, the fixed biological sample can be an aldehyde-fixed biological sample. In some embodimentexamples, the fixed biological sample can be a formaldehyde-fixed biological sample. In some embodimentexamples, the fixed biological sample can be a

formaldehyde-fixed paraffin-embedded (FFPE) biological sample. In some embodimentexamples, the fixed biological sample can be a fixed tissue section.

In some embodimentexamples, the method further includes, prior to the step of contacting the fixed biological sample with the de-crosslinking agent, a step of staining the fixed biological sample, and imaging the fixed biological sample. In some embodimentexamples, the method further includes, after the step of contacting the fixed biological sample with the de-crosslinking agent, a step of staining the de-crosslinked biological sample, and imaging the de-crosslinked biological sample. In some embodimentexamples, the staining includes the use of a histological stain. In some embodimentexamples, the staining includes the use of an immunological stain.

In some embodimentexamples, the substrate includes a slide. In some embodimentexamples, the substrate includes a bead.

In some embodimentexamples, the capture probe further includes a unique molecular identifier (UMI). In some embodimentexamples, the capture probe includes DNA. In some embodimentexamples, the capture domain includes a poly(T) sequence. In some embodimentexamples, the capture probe further includes a spatial barcode.

In one aspect, the present disclosure refers to a method of producing a de-crosslinked biological sample, the method comprising: (a) contacting a fixed biological sample with a de-crosslinking agent of compound Formula (I) or compound of Formula (II), thereby producing the de-crosslinked biological sample; wherein the de-crosslinking is performed prior to or subsequent to contacting the fixed biological sample with a substrate comprising a plurality of capture probes, wherein a capture probe comprises a capture domain.

In another aspectexample, provided herein is a method of determining a location of a nucleic acid analyte in the fixed biological sample, the method including (i) producing a de-crosslinked biological sample according to any of the methods described herein, wherein the capture domain binds specifically to the nucleic acid analyte of the biological sample, and (ii) determining (I) a sequence corresponding to the nucleic acid analyte or a complement thereof, and (II) a sequence corresponding to the spatial barcode of the capture probe or a complement thereof, and using the determined sequences of (I) and (II) to determine the location of the nucleic acid analyte in the fixed biological sample.

In another aspectexample, provided herein is a method of determining a location of a nucleic acid analyte in the fixed biological sample, the method including (i) producing a de-crosslinked biological sample according to any of the methods described herein, wherein the capture domain binds to the nucleic acid analyte of the biological sample, and (ii) determining (I) a sequence corresponding to the nucleic acid analyte or a complement thereof, and (II) a sequence corresponding to the spatial barcode of the capture probe or a complement thereof, and using the determined sequences of (I) and (II) to determine the location of the nucleic acid analyte in the fixed biological sample.

In some embodimentexamples, the nucleic acid analyte can be DNA. In some embodimentexamples, the nucleic acid analyte can be an RNA. In some embodimentexamples, the RNA can be an mRNA.

In some embodimentexamples, the method further includes extending an end of the capture probe using the nucleic acid analyte as a template.

In one aspect, the present disclosure refers to a method of determining a location of a nucleic acid analyte in a fixed biological sample, the method comprising:

(i) producing a de-crosslinked biological sample using the method described herein,
(ii) hybridizing the capture domain of a capture probe on an array, wherein the capture probe further comprises a spatial barcode, to the nucleic acid analyte of the de-crosslinked biological sample;

(iii) extending the capture domain of the capture probe using the nucleic acid analyte as a template; and

(iv) determining (I) a sequence corresponding to the nucleic acid analyte or a complement thereof, and (II) a sequence corresponding to the spatial barcode of the capture probe or a complement thereof, and using the determined sequences of (I) and (II) to determine the location of the nucleic acid analyte in the fixed biological sample.

In another aspectexample, provided herein is a method of determining the location of a protein analyte in the fixed biological sample, the method including (i) contacting a de-crosslinked biological sample on a substrate including a plurality of capture probes, wherein a capture probe of the plurality of capture probes includes a capture domain and a spatial barcode, with a plurality of analyte capture agents, wherein an analyte capture agent of the plurality of analyte capture agents includes (1) an analyte binding moiety that binds specifically to the protein analyte from the fixed biological sample, (2) an analyte binding moiety barcode, and (3) an analyte capture sequence, wherein the analyte capture sequence binds specifically to the capture domain of the capture probe, and (ii) determining (I) a sequence corresponding to the analyte binding moiety barcode or a complement thereof, and (II) a sequence corresponding to the spatial barcode of the capture probe or a complement thereof, and using the determined sequences of (I) and (II) to determine the location of the protein analyte in the fixed biological sample.

In another aspectexample, provided herein is a method of determining the location of a protein analyte in the fixed biological sample, the method including (i) contacting a de-crosslinked biological sample on a substrate including a plurality of capture probes, wherein a capture probe includes a capture domain and a spatial barcode, with a plurality of analyte capture agents, wherein an analyte capture agent includes (1) an analyte binding moiety that binds specifically to the protein analyte from the fixed biological sample, (2) an analyte binding moiety barcode, and (3) an analyte capture sequence, wherein the analyte capture sequence binds to the capture domain of the capture probe, and (ii) determining (I) a sequence corresponding to the analyte binding moiety barcode or a complement thereof, and (II) a sequence corresponding to the spatial barcode of the capture probe or a complement thereof, and using the determined sequences of (I) and (II) to determine the location of the protein analyte in the fixed biological sample.

In some embodimentexamples, the biological sample can be de-crosslinked using a de-crosslinking agent that is a compound of Formula (I) or a compound of Formula (II). In some embodimentexamples, the biological sample can be de-crosslinked using a de-crosslinking agent selected from the group consisting of compounds (1)-(18). In some embodimentexamples, the biological sample can be de-crosslinked using a de-crosslinking agent that is a compound of Formula (I). In some embodimentexamples, the biological sample can be de-crosslinked using a de-crosslinking agent selected from the group consisting of compounds (1)-(14). In some

embodimentexamples, the biological sample can be de-crosslinked using a de-crosslinking agent selected from the group consisting of compounds (1)-(11). In some embodimentexamples, the biological sample can be de-crosslinked using a de-crosslinking agent that is a compound of Formula (II). In some embodimentexamples, the biological sample can be de-crosslinked using a de-crosslinking agent selected from the group consisting of compounds (15)-(18). In some embodimentexamples, the biological sample can be de-crosslinked using compound (1).

In some embodimentexamples, the method includes, prior to (i), producing the de-crosslinked biological sample according any of the methods described herein.

In some embodimentexamples, the protein analyte can be an intracellular protein. In some embodimentexamples, the protein analyte can be an extracellular protein. In some embodimentexamples, the protein analyte can be a cell surface protein.

In some embodimentexamples, the analyte-binding moiety includes an antibody or an antigen-binding domain thereof.

In some embodimentexamples, method further includes extending an end of the capture probe using the analyte binding moiety barcode as a template.

In some embodimentexamples, the determining of the sequences of (I) and (II) includes sequencing of the sequences of (I) and (II). In some embodimentexamples, the sequencing can be high throughput sequencing.

In one aspect, the present disclosure refers to a method of determining the location of a protein analyte in a fixed biological sample, the method comprising:

(i) contacting a de-crosslinked biological sample with a substrate comprising a plurality of capture probes, wherein a capture probe comprises a capture domain and a spatial barcode, with a plurality of analyte capture agents, wherein an analyte capture agent comprises:

(1) an analyte binding moiety that binds specifically to the protein analyte from the fixed biological sample;

(2) a capture agent barcode domain comprising

_____ (i) an analyte binding moiety barcode; and

_____ (ii) an analyte capture sequence, wherein the analyte capture sequence

is complementary to the capture domain of the capture probe; and

(ii) determining (I) a sequence corresponding to the analyte binding moiety barcode or a complement thereof, and (II) a sequence corresponding to the spatial barcode of the capture probe or a complement thereof, and using the determined sequences of (I) and (II) to determine the location of the protein analyte in the fixed biological sample.

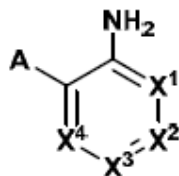
In one aspect, the present disclosure refers to a method of determining a location of a nucleic acid analyte in a fixed biological sample, the method comprising:

(a) providing an array, wherein the array comprises a plurality of capture probes and wherein a capture probe of the plurality of capture probes comprises: (i) a spatial barcode and (ii) a capture domain;

(b) de-crosslinking the fixed biological sample, wherein at least one of the de-crosslinking agent comprises one or more of:

(i) 2-amino-5-methylbenzoic acid, 2-amino-5-nitrobenzoic acid, 2-amino-5-methylbenzenesulfonic acid, 2,5-diaminobenzenesulfonic acid, 2-amino-3,5-dimethylbenzenesulfonic acid, (2-amino-5-nitrophenyl)phosphonic acid, (4-aminopyridin-3-yl)phosphonic acid, and (2-amino-5-{[2-(2-poly-ethoxy)ethyl]carbamoyl}phenyl)phosphonic acid, or a pharmaceutically acceptable salt thereof; and/or

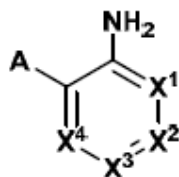
(ii) the de-crosslinking agent comprises formula (I)



(I) or a pharmaceutically acceptable salt thereof,

wherein A is $P(=O)(OH)_2$, and wherein each of X^1 , X^2 , X^3 , and X^4 is CH; and/or

(iii) the de-crosslinking agent comprises formula (I):



(I) or a pharmaceutically acceptable salt thereof,

wherein:

A is $P(=O)(OH)_2$ or $C(=O)OH$;

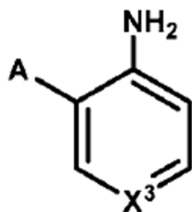
each of X^1 , X^2 , X^3 , and X^4 is, independently, CH, CR^a , or N;

each R^a is, independently, C_{1-6} alkyl, C_{1-6} haloalkyl, NO_2 , $NR'R''$, or $C(=O)NR'R''$;

each of R' and R'' is, independently, H or optionally substituted C_{1-6} alkyl; and

at least one of X^1 , X^2 , X^3 , and X^4 is N; and/or

(iv) the de-crosslinking agent comprises formula (IB):



(IB) or a pharmaceutically acceptable salt thereof,

wherein A is $P(=O)(OH)_2$; and

X^3 is CH or N;

(c) hybridizing the nucleic acid analyte of the fixed biological sample to the capture domain; and

(d) determining (I) all or a portion of the sequence of the nucleic acid analyte or a complement thereof, and (II) the sequence of the spatial barcode or a complement thereof, and using the determined sequences of (I) and (II) to determine the location of the nucleic acid analyte in the fixed biological sample.

In another [aspectexample](#), provided herein is a method of producing a de-crosslinked biological sample, the method including (a) contacting a formaldehyde-fixed paraffin-embedded (FFPE) biological sample with a substrate including a plurality of capture probes, wherein a capture probe of the plurality of capture probes includes a capture domain (b) deparaffinizing the FFPE biological sample, (c) staining the FFPE biological sample with hematoxylin and eosin, (d) pre-treating the FFPE biological sample with collagenase, a detergent, or collagenase or a detergent, and (e) contacting the FFPE biological sample with compound (1), thereby producing the de-crosslinked biological sample.

In another [aspectexample](#), provided herein is a method of producing a de-crosslinked biological sample, the method including (a) contacting a formaldehyde-fixed paraffin-embedded (FFPE) biological sample with a substrate including a plurality of capture probes, wherein a capture probe includes a capture domain (b) deparaffinizing the FFPE biological sample, (c) staining the FFPE biological sample with hematoxylin and eosin, (d) pre-treating the FFPE biological sample with collagenase, a detergent, or collagenase or a detergent, and (e) contacting the FFPE biological sample with compound (1), thereby producing the de-crosslinked biological sample.

In some embodiment examples, deparaffinizing the FFPE biological sample includes, sequentially, contacting the FFPE biological sample with xylene, absolute ethanol, about 96% ethanol, and about 70% ethanol.

In some embodiment examples, the FFPE sample can be pre-treated with collagenase at 0.2U/μL in HBSS buffer for 20 minutes at 37 °C.

In some embodiment examples, the FFPE sample can be pre-treated with 0.5% of a non-ionic detergent in TE buffer at pH 8 for 20 minutes at 37 °C.

In some embodiment examples, the FFPE sample can be contacted with 50 mM compound (1) in Tris or TE buffer for 1 hour at 70 °C.

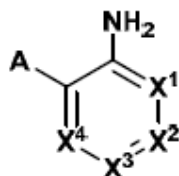
In one aspect, the present disclosure refers to a method of de-crosslinking a fixed biological tissue sample, the method comprising:

contacting the fixed biological tissue sample with a de-crosslinking agent, wherein at least one of the de-crosslinking agent is selected from the group consisting of: 2-amino-5-methylbenzoic acid, 2-amino-5-nitrobenzoic acid, 2-amino-5-methylbenzenesulfonic acid, 2,5-diaminobenzenesulfonic acid, 2-amino-3,5-dimethylbenzenesulfonic acid, (2-amino-5-nitrophenyl)phosphonic acid, (4-aminopyridin-3-yl)phosphonic acid, and (2-amino-5-{[2-(2-poly-ethoxy)ethyl] carbamoyl}phenyl)phosphonic acid, or a pharmaceutically acceptable salt thereof;

thereby de-crosslinking the fixed biological tissue sample.

In one aspect, the present disclosure refers to a method of de-crosslinking a fixed biological tissue sample, the method comprising:

contacting the fixed biological tissue sample with a de-crosslinking agent, wherein the de-crosslinking agent comprises formula (I):

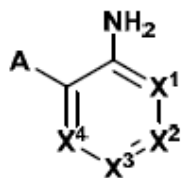


(I) or a pharmaceutically acceptable salt thereof,

wherein A is P(=O)(OH)₂, and wherein each of X¹, X², X³, and X⁴ is CH;
thereby de-crosslinking the fixed biological tissue sample.

In one aspect, the present disclosure refers to a method of de-crosslinking a fixed biological tissue sample, the method comprising:

contacting the fixed biological tissue sample with a de-crosslinking agent, wherein the de-crosslinking agent comprises formula (I):



(I) or a pharmaceutically acceptable salt thereof,

wherein:

A is P(=O)(OH)₂ or C(=O)OH;

each of X¹, X², X³, and X⁴ is, independently, CH, CR^a, or N;

each R^a is, independently, C₁₋₆ alkyl, C₁₋₆ haloalkyl, NO₂, NR'R'', or

C(=O)NR'R'';

each of R' and R'' is, independently, H or optionally substituted C₁₋₆

alkyl; and

at least one of X¹, X², X³, and X⁴ is N;

thereby de-crosslinking the fixed biological tissue sample.

Also provided herein is a kit for practicing any of the methods described herein, the kit including (a) a substrate including a plurality of capture probes, wherein a capture probe comprises a spatial barcode and a capture domain, and (b) a reagent including one or more of compounds (1)-(18). In some embodiment examples, the compound can be compound (1). In some embodiment examples, the kit can further include (a) one or more polymerase enzymes, (b) one or more wash buffers, and (c) one or more reaction buffers. In one aspect, the present disclosure refers to a kit for practicing any of the methods described herein comprising:

(a) a substrate comprising a plurality of capture probes, wherein a capture probe comprises a spatial barcode and a capture domain,

(b) a reagent comprising one or more of compounds (1)-(18),

(c) one or more polymerase enzymes,

(d) one or more wash buffers, and

(e) one or more reaction buffers.

Also provided herein are compositions that comprise or consist of compound (1), compound (2), compound (3), compound (4), compound (5), compound (6), compound (7), compound (8), compound (9), compound (10), compound (11), compound (12), compound (13), compound (14), compound (15), compound (16), compound (17), or compound (18).

Also provided herein are compositions that comprise or consist of one or more of: compound (1), compound (2), compound (3), compound (4), compound (5), compound (6), compound (7), compound (8), compound (9), compound (10), compound (11), compound (12), compound (13), compound (14), compound (15), compound (16), compound (17), and compound (18).

As used herein, the term "halo" refers to fluoro (F), chloro (Cl), bromo (Br), or iodo (I).

The term "alkyl" refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C₁₋₁₀ indicates that the group may have from 1 to 10 (inclusive) carbon atoms in it. Non-limiting examples include methyl, ethyl, *iso*-propyl, *tert*-butyl, *n*-hexyl.

The term "haloalkyl" refers to an alkyl, in which one or more hydrogen atoms is/are replaced with an independently selected halo.

The term "alkoxy" refers to an -O-alkyl radical (e.g., -OCH₃).

The term "alkylene" refers to a divalent alkyl (e.g., -CH₂-).

The term “alkenyl” refers to a hydrocarbon chain that may be a straight chain or branched chain having one or more carbon-carbon double bonds. The alkenyl moiety contains the indicated number of carbon atoms. For example, C₂₋₆ indicates that the group may have from 2 to 6 (inclusive) carbon atoms in it.

5 The term “alkynyl” refers to a hydrocarbon chain that may be a straight chain or branched chain having one or more carbon-carbon triple bonds. The alkynyl moiety contains the indicated number of carbon atoms. For example, C₂₋₆ indicates that the group may have from 2 to 6 (inclusive) carbon atoms in it.

10 The term “aryl” refers to a 6-20 carbon mono-, bi-, tri- or polycyclic group wherein at least one ring in the system is aromatic (e.g., 6-carbon monocyclic, 10-carbon bicyclic, or 14-carbon tricyclic aromatic ring system); and wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of aryl groups include phenyl, naphthyl, tetrahydronaphthyl, and the like.

15 The term “heteroaryl”, as used herein, means a mono-, bi-, tri- or polycyclic group having 5 to 20 ring atoms, alternatively 5, 6, 9, 10, or 14 ring atoms; and having 6, 10, or 14 pi electrons shared in a cyclic array; wherein at least one ring in the system is aromatic, and at least one ring in the system contains one or more heteroatoms independently selected from the group consisting of N, O, and S (but does not have to be a ring which contains a heteroatom, e.g. tetrahydroisoquinoliny, e.g., tetrahydroquinoliny). Heteroaryl groups can
20 either be unsubstituted or substituted with one or more substituents. Examples of heteroaryl include thienyl, furyl, oxazolyl, oxadiazolyl, pyrrolyl, imidazolyl, triazolyl, thiodiazolyl, pyrazolyl, isoxazolyl, thiadiazolyl, pyranyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, triazinyl, thiazolyl benzothienyl, benzoxadiazolyl, benzofuranyl, benzimidazolyl, benzotriazolyl, cinnoliny, indazolyl, indolyl, isoquinoliny, isothiazolyl, naphthyridinyl,
25 purinyl, thienopyridinyl, pyrido[2,3-*d*]pyrimidinyl, pyrrolo[2,3-*b*]pyridinyl, quinazolinyl, quinoliny, thieno[2,3-*c*]pyridinyl, pyrazolo[3,4-*b*]pyridinyl, pyrazolo[3,4-*c*]pyridinyl, pyrazolo[4,3-*c*]pyridine, pyrazolo[4,3-*b*]pyridinyl, tetrazolyl, chromane, 2,3-dihydrobenzo[*b*][1,4]dioxine, benzo[*d*][1,3]dioxole, 2,3-dihydrobenzofuran, tetrahydroquinoline, 2,3-dihydrobenzo[*b*][1,4]oxathiine, isoindoline, and others. In some
30 embodimentexample, the heteroaryl is selected from thienyl, pyridinyl, furyl, pyrazolyl, imidazolyl, isoindolinyl, pyranyl, pyrazinyl, and pyrimidinyl.

All publications, patents, patent applications, and information available on the internet and mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, patent application, or item of information was

specifically and individually indicated to be incorporated by reference. To the extent publications, patents, patent applications, and items of information incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

The term “each,” when used in reference to a collection of items, is intended to identify an individual item in the collection but does not necessarily refer to every item in the collection, unless expressly stated otherwise, or unless the context of the usage clearly indicates otherwise.

20 **DESCRIPTION OF THE DRAWINGS**

FIG. 1 shows an exemplary de-crosslinking workflow.

FIG. 3 shows spatial gene expression analysis of formalin-fixed, paraffin-embedded (FFPE) mouse spleen as analyzed by t-SNE clustering. The rendered analysis picture and t-SNE are representations of a real life picture of a mouse spleen gene expression profile and the associated t-SNE analysis. In the left panel, a frame of fiducial markers surrounds the mouse spleen sample.